OXIDANTS AND ANTIOXIDANTS IN AGING

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OVERVIEW

Considerations of natural selection suggest that life span maximization is not a significant evolutionary priority.[1] Animals in the wild must survive at least long enough to reproduce in a highly competitive environment. Survival hinges on the ability to fight or escape from predators, recover from physical trauma, tolerate toxins in food, subdue infectious microbes and optimize energy storage and expenditure to endure long periods of starvation. The need to allocate energy resources to these various challenges diverts at least some resources from maintenance functions. Among important maintenance functions is the prevention of, and recovery from, chronic, inevitable damage. Such damage can arise from a variety of non-physiologic reactions in tissues, including reactions of macromolecules with reducing sugars (glycation) and other aldehydes, oxidants, alkylation by methylating agents and spontaneous hydrolytic processes. Even if some of the products of these reactions are transient, they can exert adverse effects on organisms. Incomplete repair of damaged macromolecules could well lead to the cumulative effects that characterize aging. No single damage mechanism like oxidative damage is likely to fully explain the aging phenomenon -- an "understanding" of aging may prove to comprise a ranking of the various damage (and incomplete repair) processes that characterize life.

Animals are certainly subject to considerable oxidative stress. An early observation suggesting the possible existence of such stress was Lavosier's discovery in 1878 that high oxygen concentrations (actually only a four- to five-fold higher than atmospheric levels) are toxic. Animals suffer lung and neurological damage within a few hours and die within a few days of exposure to pure oxygen.[2] However, they can survive longer oxygen exposures if gradually adapted to increasing oxygen concentrations. Humans exposed to pressurized, i.e., hyperbaric, oxygen (HBO) suffer DNA damage within a few hours of exposure, but protection is afforded by a brief pre-exposure to HBO.[3] The discovery and characterization of the "anti-oxidant" enzyme superoxide dismutase (SOD, whose only known function is to scavenge the oxygen-derived reactive molecule superoxide (an example of a free radical molecule -- see later sections of this chapter), and the observation that virtually no air-respiring animal can survive without this enzyme have established that hazardous oxygen products are formed *in vivo*. Adaptation to elevated oxygen concentrations is consistent with the view that aerobic organisms maintain antioxidant defenses that are just sufficient for protection against hazardous oxygen derivatives under normal physiologic conditions but that can be enhanced in response to gradual increases in oxidative stress. A general theme of biologic stress responses is that low doses of some specific toxic agent elicit an adaptive response that confers protection against a relatively broad range of subsequent toxic challenges. In principle this represents an amplifying mechanism for anti-oxidant protection and suggests that certain kinds of chronic stress can exert long-term net benefits. Consistent with this idea is a recent report that chronic exposure of mice to low levels of ionizing radiation, a process that produces free radicals (see subsequent sections), increased life spans by about 22%.[4]

Although destructive oxygen-derived products like free radicals may escape scavenging by antioxidants like SOD and react with vital cellular targets, organisms have the capacity to repair damage. For example, oxidative modifications of macromolecules can be (a) partially reversed by reduction, (b) repaired by excision and replacement of damaged parts or (c)

counteracted by complete turnover. Even if toxic agents trigger the death of individual cells, multicellular organisms have some capacity to compensate for cell loss by replacing irreversibly damaged cells with new ones. Hence oxidative damage theories and, indeed, all cumulative damage theories must contend with the question of why repair is incomplete or why compensatory processes like cell renewal fail. One important consideration is that no repair system is likely to be absolutely efficient if the damage is so random that macromolecules are altered in virtually limitless ways. An oxidative damage wear-and-tear theory, which embodies the characteristic of unpredictable molecular alterations, is the free radical theory of aging. It was first proposed by Harman in 1955, essentially as follows: aging results from the deleterious effects of free radicals produced in the course of cellular metabolism.[5,6] Harman supported his theory by citing the purported detection of free radicals in biologic systems with the newly discovered technique of electron paramagnetic resonance (EPR).[7] Of historical interest is that the initial reports of free radical signals in biologic tissues were incorrect -- the observed EPR signals were an artifact of the freeze-drying methods used in sample preparation, and were not due to metabolic activity as postulated in the free radical theory.[8] Subsequently, the existence of tissue free radicals was confirmed, but concentrations were much lower than suggested by the initial reports.

The stochastic nature of free radical reactions implies that cellular surveillance systems cannot possibly recognize all of the structural or functional changes that can occur. Even with perfect surveillance of exposed damage-sensitive sites, the need to repair "hidden" damage, e.g., oxidized amino acids buried within proteins, requires a periodic turnover of macromolecules, probably with energy-wasting turnover of undamaged macromolecules. These considerations suggest that the pursuit of perfect maintenance necessarily entails substantial energy dissipation in dealing with the possibility rather than certainty of damage. From an energy optimization perspective, survival in a highly competitive natural world is simply not consistent with the high level of maintenance that would ensure maximum life spans.

The free radical theory of aging continues to generate great interest as indicated by a sampling of recent publications.[9-12] Considerable evidence supports an involvement of oxidative damage in major life-shortening diseases and hence average life span. The evidence for a pivotal role of oxidants in the antimicrobial activity of immune cells, which suggests a life-shortening effect of immune system activity in certain diseases and chronic infections, is especially compelling. However, the question of whether oxidants, or indeed any damage process, limit maximum life span, i.e., control the aging process *per se*, remains unanswered. Efforts to use predictions of the free radical theory to achieve increases in maximum life-span extension, e.g., through nutritional supplementation with natural antioxidants have failed.[13]

While there is no question that free radicals can be highly destructive, it is becoming increasingly evident that at least some free radicals and non-radical oxidants play vital physiologic roles (in addition to their involvement in immune function). The free radicals nitric oxide and superoxide and the non-radical hydrogen peroxide serve as second messengers in regulating cell growth and many other physiologic processes. Of particular interest is the role of the free radical nitric oxide in controlling mitochondrial metabolism. A

growing body of evidence argues for the likelihood that many of the adverse effects of at least some oxidants may be attributable to their disruptive effects on cell regulation rather than to overt damage.[10]

OXIDANTS

Oxidants of biologic importance are small molecules containing oxygen, referred to as reactive oxygen species (ROS). Some oxidants also contain nitrogen and are referred to as reactive nitrogen species (RNS). ROS readily oxidize cellular constituents like DNA, proteins or lipids in the most basic chemical sense, i.e. in reactions that involve one or more electron transfers from an organic target molecule to the oxidant. However some biologic oxidants, notably certain free radicals, also alter organic molecules by combining with them to form "adducts." ROS comprise a group of molecules that span a broad range of reactivities. Some ROS like the superoxide radical are relatively weak and are likely to collide with many other molecules before a reaction occurs. Others, like the hydroxyl radical, are extremely reactive and will react with virtually any organic molecule they encounter. Important biologic oxidants are listed in Table 1.

A. WHAT ARE FREE RADICALS?

Free radicals are molecular fragments containing "unpaired" electrons. They are produced when chemical bonds are broken. Considerable energy is required to break most chemical bonds so free radicals rarely occur in nature. Once formed, free radical fragments will usually react with non-radical molecules to form new free radical products or combine with other free radicals to form stable products. Even if sufficient energy is available to break bonds, the proportion of free radicals among non-radicals is usually very low. Examples of free radicals include:

- the very reactive hydroxyl radical, OH· (the dot refers to an unpaired electron)
- the moderately reactive thiyl radical, RS·
- the weakly reactive nitric oxide radical, NO·

Reactive free radicals can pose a considerable hazard to biological systems because of their unique chemistry, which distinguishes free radicals from other toxic agents. The most damaging free radicals exhibit some or all of the following reaction patterns:

- attack other molecules indiscriminately,
- initiate oxygen-consuming chain reactions, such that a single free radical effectively damages a large number of other molecules,
- cause fragmentation or random crosslinking of molecules, including vital macromolecules like DNA, enzymes and structural proteins.

B. OXYGEN

Ordinary oxygen has two unpaired electrons, i.e., O_2 can be represented as $^{\cdot}O\text{-}O^{\cdot}$. Oxygen is an example of a free radical that is normally quite stable, i.e., oxygen radicals do not combine with each other and they do not react spontaneously with combustible organic molecules. However, oxygen can be quite reactive in the presence of other free radicals. In a

free radical chain reaction this property of oxygen is expressed as an incorporation of oxygen molecules into organic free radicals ($R^{\cdot}$, $R''^{\cdot}$), converting them to hydroperoxides as follows:

$R^{\cdot} + O_2 \rightarrow ROO^{\cdot}$

$ROO^{\cdot} + RH' \rightarrow ROOH + R''^{\cdot}$

$R''^{\cdot} + O_2 \rightarrow R'OO^{\cdot}$

In this sequence of reactions oxygen combines with an organic free radical ($R^{\cdot}$) to form a peroxyl radical ($ROO^{\cdot}$), which in turn oxidizes an organic molecule R'H. Such chain reactions exert their adverse effects by chemically modifying target molecules and by consuming oxygen required for normal metabolic processes. Another highly reactive oxygen species is singlet oxygen, which can arise during free radical chain reactions. When two peroxyl radicals react with each other they do not form a stable product but decompose into a variety of fragments, including singlet oxygen.

Oxygen can be "activated" by the sequential incorporation of electrons (e^-) and protons as follows:

$O_2 + e^- \rightarrow O_2^{\cdot -}$ (superoxide radical, weakly reactive)

$O_2^{\cdot -} + e^- + 2\ H^+ \rightarrow H_2O_2$ (hydrogen peroxide, weakly reactive)

$H_2O_2 + e^- \rightarrow OH^- + OH^{\cdot}$ (hydroxyl radical, strongly reactive)

$OH^{\cdot} + e^- + H^+ \rightarrow H_2O$

A major fate of the superoxide radical is "dismutation," a reaction of two superoxide radicals with each other and with two protons as follows: $2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$. Superoxide is not an effective electron donor to hydrogen peroxide however, so reducing agents (electron donors) that produce superoxide radicals generally lead to an accumulation of hydrogen peroxide but do not produce hydroxyl radicals. Importantly, superoxide radicals can activate hydrogen peroxide to hydroxyl radicals in the presence of certain metal ions. Either iron or copper ions, loosely bound by low molecular compounds or macromolecules catalyze this activation of hydrogen peroxide in the "Fenton reaction."

C. NITRIC OXIDE

Nitric oxide ($NO^{\cdot}$) is a free radical, which like oxygen, is essentially inert towards non-radical molecules and quite reactive with many other free radicals. However, in contrast to oxygen, when $NO^{\cdot}$ reacts with an organic free radical the product of the reaction is a stable non-radical product, i.e., $R^{\cdot} + NO^{\cdot} \rightarrow RNO$. Therefore, $NO^{\cdot}$ can terminate free radical chain reactions. From a biologic perspective one of the most important properties of nitric oxide is its reactivity with the superoxide radical to produce the non-radical peroxynitrite: $O_2^{\cdot -} + NO^{\cdot} \rightarrow ONOO^-$. Peroxynitrite is a strong oxidizing agent that is capable of nitrating aromatic compounds, e.g., converting the amino acid tyrosine to nitrotyrosine. Peroxynitrite is weakly acidic such that most of this species is negatively charged at physiological pH. However, even at physiological pH, a significant fraction of peroxynitrite is in the protonated uncharged form, which can readily diffuse into and through biologic membranes. Peroxynitrite can decompose into two new free radicals – nitrogen dioxide and the hydroxyl radical. This represents an alternative mechanism to iron or copper catalysis for generating the extremely reactive hydroxyl radical.

D. ENZYME ACTIVATION OF OXYGEN

Many enzymes involved in energy metabolism have iron or other reduction/oxidation-active (redox-active) metals at their active sites. In their reduced state some of these metallo-enzymes have the potential to transfer electrons to molecular oxygen, producing superoxide radicals and hydrogen peroxide. Enzymes capable of reducing molecular oxygen to superoxide and hydrogen peroxide also include certain flavoenzymes. Generally an electron transfer from an enzyme to molecular oxygen would be an "accidental" electron transfer that the enzyme was not designed to catalyze. In at least one case, however, an enzyme has the specific function of producing superoxide radicals. That enzyme is an NADPH oxidase, which was originally found in cells of the immune system, but has more recently been detected in many other cells. Some metallo-enzymes may form complexes with superoxide, resulting in altered enzyme activities. An altered structure of enzymes with high superoxide affinities may be one mechanism whereby low superoxide levels can function as second messengers. The interaction of superoxide with some metallo-enzymes causes a release of the metal from the enzyme. Aconitase, an important mitochondrial enzyme, loses iron upon reacting with superoxide, causing not only its inactivation, but leading to the formation of hydroxyl radicals by the released iron (Fenton reaction).[14]

E. ENZYME ACTIVATION OF HYDROGEN PEROXIDE

Certain heme proteins are converted to highly oxidizing or "ferryl" species by hydrogen peroxide. These include a variety of peroxidases that are designed to exploit the oxidizing potential of hydrogen peroxide for specific molecular transformations. They also include myeloperoxidase, which is found in cells of the immune system. Myeloperoxidase activation of hydrogen peroxide leads to the formation of hypochlorous acid at physiologic chloride concentrations. Hypochlorous acid, (the active ingredient of household bleach), diffuses from the enzyme and readily oxidizes a variety of molecules, including sulfhydryl groups on proteins and low molecular weight thiols. Hypochlorous acid reacts with superoxide radicals to form hydroxyl radicals by a mechanism that does not require transition metal ions. Free amino acids are oxidized to aldehydes by hypochlorous acid. One of these reaction products is the highly toxic unsaturated aldehyde acrolein, which is formed from the oxidation of threonine. Acrolein is a highly cytotoxic molecule, which is found in tobacco smoke and arises during the decomposition of cyclophosphamide, a compound used to kill tumor cells in chemotherapy.

An important aspect of enzyme-mediated oxidant production is that of enzyme dysfunction. Enzymes that have been oxidatively modified may be inactivated or may be more likely to promote the activation of oxygen or of hydrogen peroxide. For example, an enzyme of purine catabolism is xanthine dehydrogenase, which conserves the bond energy in xanthine to produce NADH. Oxidation of thiol groups in xanthine dehydrogenase converts it to xanthine oxidase, which produces superoxide and hydrogen peroxide without conserving energy. A peroxidase may lose its substrate specificity after oxidative modification, and exert its powerful oxidizing potential on molecules it was not designed to oxidize. Oxidation of hemoglobin and myoglobin converts these ferrous oxygen-binding proteins to ferric products with peroxidase activity and no significant oxygen-binding capacity. A vicious cycle of

oxidative enzyme modification and increased free radical production could lead to substantial damage amplification under conditions of oxidant stress.

F. PRO-OXIDANTS IN FOODS AND OXIDANT-DAMAGED TISSUES

The world of plants survives by waging chemical warfare against predators. Many of the toxic agents that protect plants act by promoting oxygen activation or by reacting directly with important cellular targets like DNA. Oxygen activation in animals can be catalyzed by plant quinones that interact with reducing enzymes and divert electrons directly to oxygen. A number of plant toxins are activated by P-450 enzymes, mammalian enzymes normally involved in detoxification of a broad range of hazardous agents, to mutagenic products or interact with the P-450 enzyme system to promote superoxide formation. Other compounds, designated as quinonoids, can catalyze a non-enzymic one-electron reduction of oxygen by low molecular weight compounds in cells. An example of such a catalytic agent is divicine, which is derived from fava beans. This agent can be reduced by simple sulfhydryl compounds in the aqueous phase of cells and subsequently reoxidized by oxygen in a reaction that produces superoxide radicals. Pro-oxidants can also form in oxidant-stressed animal tissues. Among the oxidation products of uric acid, a product of purine metabolism, is alloxan, which exhibits a superoxide-generating activity similar to that of divicine. In the presence of alloxan vitamin C becomes a source of superoxide radicals.[15]

G. ANTIOXIDANTS

Antioxidants can greatly reduce the adverse impact of oxidants by: (a) intercepting oxidants before they react with vital biologic targets, (b) preventing chain reactions or (c) preventing the activation of oxygen to highly reactive products. Low molecular weight antioxidants can be maintained at high intracellular concentrations without occupying excessive cell volume and are therefore good candidates for intercepting free radicals that would otherwise target vulnerable macromolecules. To be effective such antioxidants must readily react with free radicals to form relatively harmless products. Probably the most efficacious low molecular weight antioxidant is ascorbic acid (vitamin C), which scavenges virtually all free radicals that come into contact with it. It is water-soluble and reacts with oxidizing free radicals by donating electrons or hydrogen atoms to them. An important example of such a reaction is the reaction of an antioxidant with a free radical (one-electron oxidation) product of a macromolecule like DNA or a protein. If present at sufficiently high concentrations, ascorbic acid can donate a hydrogen atom to the unpaired electron of the macromolecule before oxygen combines with it, thereby restoring its original structure. In the case of an oxidized membrane lipid ascorbic acid, in conjunction with vitamin E (see below), can either restore its structure or stabilize its oxidation product as a relatively benign *trans*-unsaturated fatty acid. An antioxidant can also affect the reaction product of a free radical addition reaction (adduct) by donating an electron to a reactive free radical intermediate that might otherwise reduce an oxygen molecule to a superoxide radical. Important antioxidants and related protective agents are listed in Table 2.

Ascorbic acid

This widely consumed nutritional supplement is converted to the virtually inert ascorbyl radical when it reacts with free radicals. Its potency as an antioxidant is illustrated by its rapid reduction of nitroxide free radicals, synthetic organic radicals that are so unreactive as to be stable in aqueous solution. Ascorbic acid also plays a direct role in a number of enzyme reactions, serving a vital function in collagen synthesis and, hence, in the maintenance of tissue structure. The ascorbyl radical does not reduce oxygen to superoxide. It is one of the few free radicals that can be observed directly in some tissue preparations with magnetic resonance techniques, which testifies to its lack of reactivity. Most animals synthesize ascorbic acid; humans and higher primates do not. Ascorbate is found in fruits and vegetables, which normally provide an adequate supply of this vitamin. A lack of dietary ascorbate can lead to scurvy, which had once been a major cause of mortality among sailors during long sea voyages. The symptoms of scurvy can be attributed to a failure to synthesize collagen without invoking a role for oxidant damage. In some tissues, the ascorbyl radical can be reduced back to ascorbate by specific enzymes. Alternatively, two ascorbyl radicals will dismutate, leading to the formation of dehydroascorbic acid. Dehydroascorbic acid is not stable in the body of an animal and has toxic properties. It can react with and chemically modify biomolecules and has been used as a diabetogenic agent in animals. It decomposes with a half life of 6.5 minutes at 37^{o}C if it is not reduced to ascorbic acid enzymatically or by a non-enzymatic reaction with glutathione (see below) or other thiols. Ascorbate may play a role in protecting membranes by donating a hydrogen atom to free radicals at the membrane-water interface. Ascorbate can exert adverse effects by acting as a pro-oxidant, e.g., in the presence of certain metal ions. It is capable of reducing loosely bound iron or copper and can thus participate in the activation of hydrogen peroxide to hydroxyl radicals. A surprising recent discovery was that ascorbate can interact with lipid hydroperoxides to produce genotoxic aldehydes by a mechanism that was previously thought to be possible only in the presence of transition metal ions.[16] This important discovery may explain some of the disappointing failures of ascorbic acid therapy in treating diseases (see later section on oxidants in disease).

An important characteristic of reactive free radicals is the tendency for reaction products to be weaker radicals than reaction precursors. A typical reaction cascade would begin with a hydroxyl radical forming less reactive carbon-centered radicals (the free electron resides mostly on a carbon atom of an organic molecule), which in turn would form even less reactive peroxyl radicals. The latter would be relatively persistent and eventually react with glutathione or ascorbate.

Glutathione

Thiols and glutathione in particular are involved in a variety of protective cell functions. Glutathione is a cysteine-containing tripeptide that is found in millimolar concentrations in most cells. The radical that is formed from glutathione (or most other thiols), referred to as a thiyl radical, is considerably more reactive than the ascorbyl radical, but will generally react with another thiol like glutathione, producing a disulfide product and a superoxide radical. The latter is generally dismutated by superoxide dismutase, so no damage results from this reaction pathway. However, thiyl radicals do react with phenolic molecules like tyrosine and therefore have significant destructive potential. A specific enzyme, glutathione reductase,

regenerates glutathione from disulfide products, using NADPH generated by the hexose monophosphate pathway or by isocitrate dehydrogenases as the reductant.

Vitamin E

Vitamin E is another widely consumed vitamin. The E vitamins are a group of hindered phenolic, hydrophobic antioxidants that can donate hydrogen atoms to lipid radicals in membranes. They are also known as tocopherols, comprising a group of phenols with different substituents on a phenolic ring attached to a long hydrophobic chain that intercalates into membrane lipid domains and binds to certain proteins. After donating a hydrogen atom to a free radical, the vitamin E radical, designated as the tocopheroxyl radical, is too weak to react even with a highly unsaturated fatty acid. Moreover, the α-tocophoroxyl radical differs from simple phenolic radicals in not forming radical-radical products (dimers). Thus vitamin E is a highly effective terminator of chain reactions in membranes. A number of reducing agents, including vitamin C, can react with the tocopheroxyl radical to regenerate vitamin E. Therefore, vitamin E usually acts as a catalytic agent that transfers reducing power derived from cellular metabolism to membrane free radicals.

The most common form of vitamin E in nutritional supplements is α-tocopherol. As noted above, it is the most effective antioxidant of the tocopherols, because the α-tocopheroxyl radical is highly resistant to reactions other than hydrogen-atom oxidation. Interestingly, γ-tocopherol may be more potent in protecting humans from degenerative diseases. This effect appears to be related to its inhibition of an enzyme (cyclooxygenase 2) involved in inflammation rather than to a free radical scavenging effect.[17] As such the vitamin is acting in much the same manner as aspirin in reducing risks of cardiovascular disease. Major commercial vitamin formulations consist of α-tocopherol. On the other hand, food sources contain mostly γ-tocopherol. Of concern is that α-tocopherol can exert an inhibitory effect on γ-tocopherol activity.

III. OXIDANTS AND ANTIOXIDANTS IN CELLS AND ORGANELLES

A. METABOLISM

A key element of the free radical theory of aging is the notion that metabolism spawns destructive free radicals. The long-known observation that animal life spans correlate roughly with inverse specific metabolic rate led to the suggestion that free radicals were responsible for aging because free radical production was assumed to be a direct function of metabolic rate. However, our current understanding of oxidative metabolism argues against such a simple view. About 90% of the oxygen consumed by animals is utilized by mitochondria in a harmless process that directly yields water.[18] It is not known what fraction of normal mitochondrial oxygen consumption *in vivo* comprises ROS production, but the fraction is likely to be much less than the 2% observed in isolated organelles and cells in air-saturated solution. Superoxide production is a function of the state of reduction of the respiratory chain, not of metabolic rate. Therefore mitochondrial oxidant production *in vivo* will vary depending on tissue oxygen concentrations, concentrations of reducing substrates,

ATP utilization and the presence of electron transport inhibitors (see next section), but not necessarily the rate of oxygen consumption. Of interest is the potential effect of the general nutritional status on the mitochondrial redox potential. Recent studies on the effects of high glucose concentrations on cultured epithelial cells have shown that large increases in glucose concentration elicit a substantial increase in oxygen consumption, hydrogen peroxide production, advanced glycation end (AGE) product formation and accumulation of the glucose reduction product sorbitol.[19] Conceivably, large fluctuations in blood glucose levels rather than mean energy intake, are key determinants of oxidative damage.

Several considerations other than mechanisms of oxidant production argue against the idea that aging is governed by metabolic rate. These include the beneficial effects of exercise, analyses of the aging rates of organs exhibiting widely differing metabolic rates (e.g., brain vs. muscle) and animal species comparisons (ten-fold difference in life spans between bats and mice having the same specific metabolic rates).

B. HOMEOSTASIS

Homeostasis, e.g., the maintenance of a well-controlled redox environment, is necessary for the proper functioning of all organisms. Animals can tolerate a range of tissue oxygen concentrations, as exemplified by a range of well over an order of magnitude between intensive exercise and rest. This range is limited however, as shown by the toxicity of pure and pressurized oxygen (in the absence of gradual adaptation). At the other extreme, complete lack of oxygen as in ischemia, leads to tissue damage when oxygen becomes available again, as documented in numerous publications on "reperfusion injury." Intolerance to extremes of oxygen concentrations can be reconciled with a straightforward model of oxidant production. Assuming that superoxide radicals are produced by random collisions of "autoxidizing" reducing agents with molecular oxygen, the rate of superoxide production would be directly proportional to the oxygen concentration and to the concentration of the autoxidizable reducing agents (e.g., certain quinols and semiquinones). Hence it would be expected that exposure to pure oxygen would be associated with high superoxide burdens. On the other hand, in the absence oxygen, the concentration of autoxidizable reducing agents would accumulate. Upon reoxygenation of the tissue, there would be a "burst" of superoxide radicals and superoxide-derived oxidants.

Although free radicals are clearly produced during normal oxidative metabolism, the fact that animals survive for years indicates that the elaborate antioxidant defensive systems and repair processes substantially neutralize the threat. Homeostasis implies that cells adapt to increases in oxidant levels by augmenting their responses to these threats. Antioxidants alone cannot confer complete protection, as indicated by a multiplicity of repair and turnover processes. Repair systems include specific enzymes for regenerating oxidized proteins with metabolic reducing power, a variety of proteases that break down oxidatively damaged proteins to amino acids that can be used for synthesizing new proteins and a host of DNA repair enzymes, including enzymes that repair specific hydroxyl radical adducts of DNA bases.

C. MITOCHONDRIA, PEROXISOMES AND ENZYMES

Although it is known that the mitochondrial respiratory chain operates as an array of enzymes that perform electron transfer reactions very similar to those occurring in simple chemical free radical reactions, the structure of these enzymes ensures that few or no untoward chemical reactions occur. In particular, no release of active oxygen molecules occurs from the site of oxygen binding -- cytochrome oxidase. However, despite the tightly controlled flow of reducing equivalents through the respiratory chain, some leakage of reductants from mitochondria appears to occur at sites other than cytochrome oxidase. This can be seen in a release of superoxide radicals or hydrogen peroxide in studies with isolated mitochondria, submitochondrial particles and cell suspensions. Blockage of electron flow by a variety of specific electron transport inhibitors causes part of the mitochondrial electron transport chain to become highly reducing, thereby increasing superoxide production. Electron flow is "coupled" to ion gradients (expressed as the electrochemical potential) and the ATP/ADP ratio. Because of this coupling, an increase in the magnitude of the electrochemical potential or the ATP/ADP ratio also increases superoxide production. A "safety valve" that can prevent an excessive mitochondrial electrochemical potential comprises uncoupling proteins (UCP's) that induce proton leaks in conjunction with organic anions, e.g., free fatty acids. Evidence for this concept is the report that ROS production is elevated in knockout mice lacking the mitochondrial uncoupling protein UCP3.[20] Another safety valve may be the non-specific permeability pore of the inner mitochondrial membrane. Opening of this pore is reversibly induced by increases in ROS; when ROS levels decrease the pores close. Failure to control the production of ROS by uncoupling proteins or pores has been proposed to cause selective degradation of vulnerably subpopulations of mitochondria, which has been referred to as "mitoptosis."[21] A release of intramitochondrial proteins as a result of extensive formation of non-specific pores is known to orchestrate apoptosis. Mitochondria have been called the "poison cupboard" of the cell because they contain many of the agents involved in apoptosis.[22] Knockout mice lacking the means to export ATP from mitochondria and thus not able to maintain appropriate ATP/ADP ratios have been reported to produce high levels of ROS and to exhibit an accelerated accumulation of mitochondrial DNA damage.[23]

It has recently been reported that mitochondrial respiration may be regulated by nitric oxide.[24] Nitric oxide binds reversibly to cytochrome oxidase, thereby inhibiting oxygen binding and hence respiration. At the oxygen concentrations found in tissues, respiration is substantially inhibited by nitric oxide in the submicromolar concentration range. Mitochondria contain nitric oxide synthase. Partial inhibition of respiration by nitric oxide produced by this enzyme could exert beneficial effects, e.g., by preventing steep oxygen concentration gradients across cells.[25] On the other hand, substantial blockage of electron flow by nitric oxide would render components of the electron transport chain highly reducing, thus stimulating superoxide production. This suggests that exposure of mitochondria to high nitric oxide levels would lead to significant peroxynitrite-mediated damage compounded by the adverse effects of an inhibition of oxidative phosphorylation.

Other organelles and some soluble enzymes can cause both univalent and divalent oxygen reduction. Of possible significance as a risk factor in fat consumption is beta oxidation of fatty acids in peroxisomes. Estimates of hydrogen peroxide production by

isolated subcellular fractions in pioneering experiments conducted in the laboratory of Britton Chance are set forth in Table 3.[26] These classic experiments demonstrated that a variety of potential sources of active oxygen exist in most cells. However, the relevance of these and many subsequent studies to *in vivo* oxidant production remains obscure because isolated organelles are generally damaged and because interpretations of *in vitro* studies have usually failed to take into account that tissue oxygen concentrations are generally much lower than those found in laboratory suspensions of cells or subcellular fractions. Non-peroxisomal enzymes that generate ROS as normal metabolic products include monoamine oxidase and some P-450 isoenzymes. Monoamine oxidase is located in the outer mitochondrial membrane and may be a major source of hydrogen peroxide in some tissues.[27]

D. CELLS OF THE IMMUNE SYSTEM

Cells of the immune system destroy invasive microorganisms by mechanisms that include reactive oxygen molecules. The destruction of invasive microorganisms occurs in part by the action of the potent membrane-permeable oxidants hypochlorous acid and protonated (i.e. uncharged) peroxynitrite. Stimulation of neutrophils and macrophages, ultimately triggered either by cell surface antigens on invasive microbes or on oxidatively damaged macromolecules at the surfaces of host cells, leads to an "oxidative burst," comprising reduction of oxygen to superoxide by a membrane-bound NADPH-dependent enzyme and production of nitric oxide by an inducible nitric oxide synthase. As noted previously, superoxide and nitric oxide combine spontaneously to form peroxynitrite. Hydrogen peroxide that results from superoxide dismutation combines with chloride ions within the immune cells in the myeloperoxidase reaction to yield hypochlorous acid. The powerful oxidants released by the immune cells oxidize a variety of intracellular target molecules in bacteria or other invasive microbes to exert their cytotoxic effects. However, the cells of the host organism, including both tissue and immune cells, are not spared from the destructive effects of the oxidants. It must be considered that immune activity can be a major source of oxidant damage to animals. Oxidative damage, particularly uncontrolled lipid peroxidation, generates chemotactic factors that attract leukocytes to areas of high free radical activity, compounding the effects of the initial oxidative damage. Such damage-amplifying processes are probably important factors in pulmonary damage associated with emphysema and pollutant exposure and in atherosclerosis. Oxidants generated by cells of the immune system are likely to play a role in the impact of many diseases and thus very likely contribute to a shortening of mean life spans.

A possible involvement of free radicals in aging is outlined schematically in Fig 1. This scheme identifies the major sources of superoxide radicals and other oxidants, protective systems that remove these oxidants and their major cellular targets. The fundamental idea of all wear-and-tear theories of aging, including the free radical theory, is that some damage is not repaired and that damage gradually accumulates, culminating in death. While oxidized lipids can be replaced, and most protein and DNA damage can be repaired, some damage is not be repaired and accumulates with age.

E. WHAT IS THE OXIDANT BURDEN?

An effect of oxidants on the regulation of cell growth is seen at very low concentrations compared to overt oxidative damage effects, such as those seen in necrosis (Table 4).[28] Tissue oxygen concentrations are low (typically about 40 μM), most of the oxygen is normally consumed by mitochondria and antioxidant systems rapidly scavenge reactive oxygen species. Background levels of ROS under normal physiologic conditions must be low enough to allow cell signaling to function properly, i.e., the communication network involved in cell regulation cannot be distorted by the "noise" of random free radical reactions. These considerations imply that ROS levels must be very low under normal physiologic conditions and that elevated levels of ROS are likely to exert their adverse effects primarily on cell signaling. Also, to the extent that mitochondrial respiration is inhibited by nitric oxide, the background levels of this radical must be low enough to allow for an adequate maintenance of ion gradients and the generation of ATP.

It seems plausible that the highest oxidant burdens would arise from the effects of activated cells of the immune system on host tissues. Conceivably, the destructive effects of macrophage oxidants would be compounded by oxygen depletion during the oxidative burst. Membranes of host tissue cells in close proximity to macrophages would be especially vulnerable to oxidative damage.

E. TARGETS OF FREE RADICAL AND NON-RADICAL OXIDANTS

1. DNA

Probably the most important free radical targets are nuclear and mitochondrial DNA; debate continues on the relative vulnerabilities of these two targets. Addition of the hydroxyl radical to a double bond in a DNA base can lead to a large variety of hydroxylated products.[29] Reactive nitrogen species can nitrate or deaminate DNA bases. Free radical attack on the DNA backbone or chemical modification of bases can produce strand scission. Radical-radical reactions can lead to crosslinking either among segments of DNA or between DNA and proteins.

One DNA damage product that has received much attention is the hydroxylated nucleoside 8-oxo-deoxyguanosine (8-oxo-dG), which can arise from hydroxyl radical attack on deoxyguanosine (dG). This product can be detected with high sensitivity, which has facilitated extensive DNA damage measurements in a variety of cells and organisms. The measured amount of the oxidized base in extracted DNA is presumably a "steady state" fraction of the number of bases that have suffered oxidation, since it is known that at least one DNA repair enzyme specifically excises this damaged base so that it can be replaced by a new one. Unfortunately, 8-oxo-dG can easily arise during the difficult and lengthy workup procedures that precede the quantification of this damage product. Much of the literature on DNA oxidation seems marred by artifacts that led to overestimates of the abundance of 8-oxo-dG.[30] A recent improvement in methodology has suggested that detectable levels of 8-oxo-dG are formed in at least some animal tissues. In rat liver, the ratio of 8-oxo-dG to dG in nuclear DNA was reported to be about one in a million.[31] It is troubling however, that measurements of 8-oxo-dG in cultured HELA cells have consistently yielded much lower levels of this oxidation product than were observed in rat liver.[31,32] The possibility of

artifactual DNA oxidation during liver homogenization cannot be excluded and it seems likely that further improvements in methodologies could lead to significant reassessments of the extent of DNA damage *in vivo*.

Non-radical oxidants and adduct-forming agents, including products of lipid peroxidation can compound the oxidative burden on DNA. Among the reactive species that can attack DNA bases are peroxynitrite, malondialdehyde, and products derived from acrolein and 4-hydroxy-nonenal.[33]

Base methylation plays an important regulatory role in gene expression and in the differentiation that characterizes multicellular organisms. Aging is associated with a change in the pattern of base methylation and dedifferentiation. The resulting organismal dysfunctions may explain some of the phenomenology of the aging process.[34] The repair of oxidant-modified methylated bases may lead to a loss of normally methylated bases,[35] suggesting one mechanism whereby free radicals could exert adverse epigenetic effects.

2. Proteins

As already noted, some amino acids are readily oxidized. Cysteine reacts with many free radicals and with hydroperoxides to form products that react with thiols to produce disulfides. Disulfides can subsequently be reduced again to repair the lesion, indeed, to the extent that disulfides are resistant to further oxidation or chemical modification by aldehydes, the formation of protein mixed disulfides with glutathione may serve a protective function under oxidative stress. Many amino acids may be irreversibly oxidized by free radicals that are much less reactive than the hydroxyl radical. The reactive amino acids include histidine, tryptophan, and tyrosine. Tyrosine oxidation by free radicals can lead to bityrosine, a fluorescent free radical damage marker that is implicated in irreversible protein crosslinking.

As noted earlier, heme proteins, including hemoglobin and myoglobin, react with hydroperoxides to form highly oxidizing ferryl species that can produce amino acid radicals within the heme protein or oxidize nearby molecules, e.g., initiate free radical chain reactions in membranes. Activation of hydroperoxides by heme proteins can also exert its damaging effects more indirectly, by inducing a release of iron from oxidatively cleaved porphyrins. The released iron can subsequently catalyze hydroxyl radical production or other reactions at iron binding sites. Among the site-specific reactions of protein-bound iron, the oxidations of histidine, proline and arginine side chains have been cited.[36-38]

Although most oxidized proteins are either repaired or degraded and replaced by new ones, these processes are not perfect and some altered proteins may persist for considerable periods, particularly in energy-deficient cells. A dramatic example of a persistent altered protein is seen in brain myelin protein. Racemized amino acids. i.e., amino acids that have undergone an inversion from the normal L form to the mirror-image D form, accumulate in this protein throughout life (however, amino acid racemization is not thought to be a free radical process), indicating that some altered proteins are not turned over significantly during the course of an animal's life time.

Oxidatively modified individual proteins are selectively degraded in mammalian cells by proteasomes.[39] However, when oxidative attack causes protein crosslinking, the aggregated proteins are resistant to proteasome degradation[40] and, indeed, appear to bind irreversibly to proteasomes, thereby inhibiting them.[41] A decline in proteasome activity with age has been invoked to explain the accumulation of various polymerized age pigments. In some cells this accumulation can be quite substantial, e.g., 75% of the volume of large motor neurons in human centenarians may be occupied by fluorescent polymeric deposits.[42] It is likely that non-oxidative crosslinking reactions including glycation-mediated reactions contribute to the accumulation of age pigments.[43]

The catalytic activity per unit of protein antigen decreases with age. An accumulation of oxidatively modified non-functional or dysfunctional proteins, which are not recognized or are turned over at an inadequate rate, appears to play an important role in aging.[36]

3. Membranes

Most cell membranes contain polyunsaturated fatty acids that are highly susceptible to free radical oxidation. As long as vitamin E is present, the extent of free radical oxidation is controlled because the vitamin prevents or limits the occurrence of chain reactions. Depletion of vitamin E can lead to considerable oxidation of membrane polyunsaturated lipids, with adverse consequences that can include major alterations of membrane structure, release of lipid oxidation products, including cytotoxic molecules like malondialdehyde, hydroxynonenal and acrolein, and an increase in permeability to ions. Lipid radicals react avidly with oxygen and membrane free radical chain reactions can potentially create an oxygen deficit with a concomitant loss of oxidative phosphorylation. The reduction of hydroperoxides by glutathione peroxidase consumes cellular reducing power, which could also contribute to energy depletion.

IV. SUSCEPTIBLE TISSUES AND ORGANELLES

Because the cell populations in different tissues are likely to vary in their redundancy, regenerative capacity, free radical production rates, antioxidant defenses, and DNA repair rates, some tissues may be particularly sensitive to free radical damage, and these could determine aging rates in the organism.

A. NEURONAL TISSUE

Metabolically active, non-dividing cells like the neurons could well be the most susceptible targets of oxidative damage. Indeed, there is a loss of neurons with age, but such loss appears to be species-specific and to be a function of the tissue location. The surprising durability of neuronal tissue despite its large oxygen utilization appears, at first sight, to be incompatible with free radical involvement in age-related functional declines. However, a large portion of the oxygen utilization is in axons and terminals that are physically removed from sensitive and non-renewable targets like the nuclear genome. Moreover, neuronal mitochondria do not appear to produce superoxide radicals at a site that is a major source of these

radicals in other cells. Finally, considerable cell redundancy may prevent functional deficits from becoming clinically significant. As a rule, more than 40% cell loss is required in a neuronal system before functional loss in the central nervous system is apparent.

B. RADIOSENSITIVE TISSUES

Clinical experience with tumor irradiation has identified a number of tissues that are highly sensitive to free radical attack. The majority of these radiosensitive tissues have rapidly dividing cells whose DNA would be expected to be more vulnerable to oxidative attack than that of non-dividing cells. Among these rapidly dividing cells are those of the hemopoietic system, gastrointestinal tract and skin. However, some non-proliferating cells also exhibit high radiosensitivity, e.g., cells of the salivary gland. Interestingly, a recent analysis of intestinal radiation damage in mice has shown that radiation-induced apoptosis is responsible for the adverse physiologic consequences of irradiation in this tissue. Inhibition of apoptosis by pharmacological agents or genetic manipulations greatly reduced tissue damage, demonstrating that the direct effects of free radical reactions did not play a major role in the damage caused by the irradiation.[44]

C. MITOCHONDRIA

Mitochondrial dysfunction and depletion occurs in aging postmitotic cells and may prove to be a major factor in aging. Miquel and his colleagues have postulated that mitochondria are the "Achilles heel" of postmitotic cells. Mitochondrial DNA: (a) is not protected by a coat of histone proteins, (b) is in close proximity to sites of oxygen radical production and lipid peroxidation, and (c) may have an inadequate DNA repair system.[45] However, because many mitochondrial proteins are encoded by the nuclear genome and are sensitive to the cellular environment (ions, substrates) and even hormonal stimulation (viz. thyroid hormone effects on mitochondrial replication), it is difficult to unambiguously dissociate intrinsic mitochondrial damage from extrinsic factors.

V. SYSTEMIC EFFECTS

One of the most obvious characteristics of aging is the gradual decline of physiologic integrity, including the progressive impairment of neurological, immunological, humoral and metabolic function. Evidence for free radical involvement in all of these debilitating processes is growing. However, organelle and cellular redundancy and renewal may be able to compensate for the damage sufficiently to sustain function. Thus, the epithelia lining the digestive tract and skin, the blood cells and the liver may be extensively damaged and yet regenerate on a regular basis. In these renewing cell populations, the principal danger of damage appears to be neoplastic transformation.

Because aging is characterized by an increased incidence of infectious diseases one might expect immunosenescence to play a crucial role in physiologic aging. Declines in immune function begin relatively early and appear to be heavily dependent on thymic involution and selective T cell aging. Immunological declines can be, at least partially, reversed by thymic grafts or thymic hormone therapy, but complete restoration requires an additional

young bone marrow graft.

Evidence for free radical involvement in immunosenescence includes a selective vulnerability of the immune system to radiation and other free radical-generating agents. T cells, which age more rapidly than B cells, are reported to be more vulnerable to oxygen radicals and to accumulate more lipofuscin; treatment of aging mice with the antioxidant 2-mercaptoethanol delays the accumulation of T cell lipofuscin and the decline of immune function with age, and increases the mean life-span.

The thymus may also be selectively vulnerable to free radical damage. The first age-related loss in size (thymic involution) can be ascribed to the loss of the most radiosensitive (cortical) lymphocytes. The medullary epithelial cells that secrete thymic hormones are heterogenous and the early loss is again that of the most radiosensitive cells, which are active metabolically, require vitamin C for their secretory activity and appear to accumulate intrinsically autofluorescent substances (age pigments). Although there are probably developmentally programmed cellular and hormonal controls of thymic involution, significant oxidative damage seems to be involved as well.

Many antioxidants are immune stimulants and will enhance immune function both *in vivo* and *in vitro* with both young and old cells. Part of this effect appears to be due to the maintenance of reduced sulfhydryl groups. Although antioxidant treatment can boost immune function at any age, it does not markedly reduce the rate of basic aging of the immune system.

VI. MODULATION OF LIFE SPANS: ROLE OF OXIDANTS

A. IONIZING RADIATION

Considerations of parallels between ionizing radiation and metabolic processes led to the articulation of the free radical theory of aging. However, experimental studies on the effects of irradiation on animals have failed to demonstrate a life-shortening effect that could be resolved from disease. Considerable effort has been devoted to the study of the health effects of ionizing radiation, prompted largely by the advent of nuclear weapons, the occurrence of fatal accidents in nuclear industries and the utility of treating tumors with irradiation. Because of these motivations, many animal studies have focused on relatively high dose exposures or the on effects of inhaled or ingested radionuclides. It was found that high-dose irradiation causes leukemia and other cancers and that susceptibility to radiation-induced disease varies substantially among animals, e.g., susceptibility is in the order: mice>beagle dogs>burros.[46] An examination of post-irradiation survival data argues against any impact of irradiation on maximum life spans; consistent with the view that the irradiation treatments increased diseases other than cancer but did not alter aging rates *per se*. Importantly, the bulk of the early literature on life-span effects of irradiation provides no information on the possible protective effects of gradual adaptation. Moreover, the typical irradiation doses were large, comprising exposures delivered during short periods that were comparable to or greater than the background levels that the animals would accumulate during their entire

lifetimes. Relatively recently studies of the effects of very low-level chronic irradiation have been reported. These studies suggest a completely different effect of low-level irradiation than would be inferred by simple extrapolations from high-level data.[4] In seeming contradiction to the predictions of the free radical theory of aging, at least seven publications have reported an increase in life spans of mice exposed to chronic free radical stress induced by low-level ionizing radiation (references cited by Caratera etal.[4]).

It can be argued that ionizing radiation is not an appropriate model for testing the free radical theory of aging. Most importantly, it is not possible to accurately mimic endogenous oxidative damage with ionizing radiation. The energy of a given photon or particle of ionizing radiation (tens of thousands to millions of times greater than the energy of the O-H bond in water) greatly exceeds the energy of any metabolic process. Rapid dissipation of this energy gives rise to concentrated clusters of highly reactive free radicals that can produce multiple reactions in a macromolecule like DNA. Multiple free radical lesions in DNA create the potential of crosslinking and multiple strand scissions, which are much more likely to be irreparable than would be single lesions. Such multiple lesions would also be more likely to lead to cancer than would the relatively rare events produced by metabolic oxidants. Lowering the dose of ionizing radiation only changes the frequency of occurrence and not the clustering of free radicals. Although the radicals produced by radiation undoubtedly can damage DNA and other important biologic targets, chronic low-level irradiation may trigger an adaptive response whereby protective and repair processes are increased. However, such an adaptive response would have to elicit a broadly protective stress response to explain its purported life-span extending effects.

B. CALORIC INTAKE

The most dramatic extensions of life-spans in animals have been achieved by dietary manipulations, which have achieved increases in maximum life spans of up to 50% by the imposition of "caloric restriction." The efficacy of this regime in prolonging life spans has led to the suggestion that a decrease in metabolic rate by lowering calorie intake lowered the rate of oxidant damage. However, caloric restriction leads to lowered weight, and this must be taken into account. Conflicting results have been reported when the anti-aging effects of food restriction were analyzed in terms of specific metabolic rates.[11]

The term caloric restriction is an unfortunate misnomer. *Ad libitum* fed rats should be viewed as obese[11] and much of the literature on caloric restriction should perhaps be viewed as literature on "body mass vs. health and longevity." As is well known for humans, high calorie intake correlates with obesity and predisposes to disease and premature death. Risk of premature death in humans is not confined to the grossly overweight but exists over a broad range of body weights.[47] Body weights of rodents are a direct function of caloric intake.[11] Human mortality risk (inverse of mean life span) and the inverse of rodent life spans (mean and maximum) increase as smooth, non-linear functions of body mass. In the context of an oxidant damage mechanism of aging, the correlation of weight with life span could be explained if one could establish a link between calorie consumption and oxidant damage. One such link has recently been suggested by studies of tissue-cultured bovine aortic

endothelial cells. High glucose in the culture medium correlated with high oxidant production and protein glycation, while also leading to increases in several manifestations of diabetic disease.[18]

C. MUTATIONS IN RODENTS AND OTHER AGING MODELS

Aging model studies have identified a number of mutated species that outlive wild-type organisms, consistent with the view that natural selection has not prioritized maximum life spans (Table 5). Most of these studies lend support to a significant role of oxidants in determining the rate of aging. Probably most relevant to human aging are studies of mutant mice that showed a strong correlation between increased resistance to oxidative stress and longevity.[52] Of interest is the observation that the mutated mice achieved increased life spans in the absence of caloric restriction effects.

Genetic manipulations of antioxidant systems have made important contributions to an understanding of oxidative damage in aging and disease (Table 6). The importance of MnSOD is underscored by the failure of an overexpression of CuSOD to compensate of its deletion. A diversity of hydrogen peroxide scavenging systems is suggested by the minimal impact of a deletion of the major glutathione peroxidase enzyme GSH-Px1. Similarly, a multiplicity of systems of controlling adverse effects of transition metal release is suggested by the minimal effects of metallothionein deletion or overexpression. The dramatic impact of deleting heme oxygenase HO-1 argues for a potent antioxidant role of this enzyme.

D. GENETIC DISORDERS

In principle, an analysis of genetic disorders, involving defects in antioxidant defenses, could resolve whether the free radical theory of aging is valid. Unfortunately, although several examples of human mutations in one or more of the protective enzymes or antioxidant vitamins are known, including individuals with reduced levels of catalase, glutathione (GSH), or GSH peroxidase, or defective vitamin E absorption, none of these show signs of accelerated aging that can be clearly distinguished from pathology.[13]

Conversely, several genetic syndromes that exhibit some features of accelerated aging, the so-called "segmental progeroid syndromes", exhibit damage that could be consistent with increased free radical damage. These include Down's Syndrome, Ataxia Telangiectasia, Cockayne's syndrome, and, possibly, Werner's syndrome, which exhibits genetic instability, yet has normal levels of SOD, GSH peroxidase, and radiation-induced repair. The molecular basis of these "geromimetic" diseases may involve accelerated rates of chromosome breakage and the diseases frequently exhibit radiation sensitivity that suggests a high susceptibility to free radical damage.

Fanconi's anemia also exhibits characteristics of oxygen radical damage, but the pathology is so severe that death occurs in infancy or early childhood, and the only sign of an age-related effect is increased malignancy. Devastating genetic deficiencies of this type do not permit normal development and, frequently, disease-linked premature death precludes

distinguishing symptoms of accelerated aging from pathology.

Down's syndrome is characterized by a 50% elevation of the copper- zinc SOD (Cu/Zn-SOD) above normal levels of this enzyme. This increased protection against superoxide radicals fails to confer anti- aging benefits; indeed, some aspects of age-related pathology, notably senile dementia, are accelerated. There are conflicting reports concerning altered brain lipofuscin accumulation, an in vivo index of lipid peroxidation. In vitro lipid peroxidation appears to be accelerated. In patients with trisomy 21, red blood cells are abnormally sensitive to lysis in the presence of paraquat, a molecule that causes an increase in cellular superoxide production. Fibroblasts from trisomy 21 sufferers exhibit enhanced lipid peroxidation. The apparent increase in free radical damage under conditions of elevated Cu/Zn-SOD levels can be explained by a concurrent decrease in manganese SOD (Mn-SOD) levels in some tissues. In patients with monosomy 21 ("21q- or anti-Down's syndrome") cells possess only 50% of normal Cu/Zn-SOD but normal Mn-SOD. While these patients suffer from developmental abnormalities and poor survival, there are no obvious signs of accelerated aging.

The difficulty of dissociating disease from aging has been a major obstacle in exploiting genetic analyses to resolve aging mechanisms. Nevertheless, the available genetic data suggest that biological concentrations of catalase, vitamin E, glutathione and glutathione peroxidase are probably not critical determinants of rates of aging, while levels of superoxide dismutase and the overall oxygen radical defensive capacity (viz., whole body radiation) may be important factors.

E. SPECIES COMPARISONS

It has often been argued that metabolic rate determines life span, based largely on the observation that the specific metabolic rate (rate of calorie consumption per unit weight) of groups of animal species is inversely proportional to the maximum life span within different groups of animals (Table 7).[8] This correlation can also be expressed as follows: the lifetime oxygen (or energy) allocation per unit weight is fixed for any animal in that group. If one assumes that free radical production is proportional to the rate of aerobic metabolism, then the association between metabolic rates and life spans can be rationalized in terms of the free radical theory of aging. Alternatively, it can be argued that oxygen is inherently toxic and the cumulative effects of oxygen toxicity correlate directly with the total amount of oxygen that an animal is exposed to during its lifetime. The distinction between these considerations may be important in that metabolic rate is determined largely by normal mitochondrial metabolism, whereas oxygen toxicity is likely determined by abnormal metabolism.

Studies of free radical production by isolated mitochondria suggest that altered free radical production may be a more significant factor in aging than impaired antioxidant protection. Sohal and his collaborators have shown that hydrogen peroxide production by mitochondria correlates with age and species life-span and that antioxidant enzymes do not exhibit consistent correlations. They suggest that altered free radical production is a key determinant of maximum attainable life span and that variations in antioxidant enzymes exert relatively little influence.[62-64]

F. CIGARETTE SMOKING

Statistics on cigarette smoking may be regarded as the largest database on a human carcinogen. Several carcinogenic agents, including tobacco-specific carcinogens, have been identified in cigarette smoke.[65] Smoking is associated with numerous other human pathologies including cardiovascular disease.[66] Relatively little is known about mechanisms responsible for the association of cardiovascular disease with smoking. Cigarette smoke is known to contain free radicals, including nitric oxide in the gas phase and relatively persistent polymeric free radicals in the tar phase. The possible involvement of free radicals in the adverse health effects of cigarette smoke has long intrigued investigators and has been cited to explain chemical modifications of biomolecules.[67] The positive correlation of smoking with major life-shortening diseases implies that cigarette smoking reduces mean life expectancy but not necessarily maximum life span.

One of the factors complicating an assessment of the involvement of free radicals in the damaging effects of tobacco smoke is antioxidant depletion.[68,69] Tobacco smoke contains acrolein, which reacts with thiols like glutathione. It inactivates enzymes whose function involves thiol groups.[70] A role of thiol destruction in tobacco smoke carcinogenesis has been proposed in the "thiol defense hypothesis."[71] Depletion of free radical scavengers may not be a radical-induced process, but could exacerbate free radical damage. Thus antioxidant depletion could be sufficient to explain an accelerated formation of free radical damage markers.

G. DIETARY ANTIOXIDANTS

Dietary supplementation with antioxidants in a variety of animal aging models has shown that, while the mean life span is increased, there is no significant increase in the maximum life span.[13] Side effects of antioxidants must also be considered, e.g., possible caloric restriction effects associated with foul-smelling food additives like 2-mercaptoethylamine. Another potential problem with dietary antioxidants in whole animals is a possible lack of specificity. For example, antioxidants are claimed to enhance immune function, and some phenolic antioxidants like 2(3)-tert-butyl-4-hydroxy-anisole (BHA) induce quinone reductase and UDP-glucuronyl transferase systems and hence may accelerate the removal of potentially mutagenic chemicals that could affect aging by non-radical mechanisms. Two other synthetic antioxidants, BHT and ethoxyquin, have been shown to induce hepatic enzymes. Thus, increased life span that correlates with antioxidant feeding may be due to effects other than free radical quenching.

The accumulation of lipofuscin is increased with vitamin E deficiency and decreased with dietary antioxidants. However, there is no convincing evidence that lipofuscin accumulation correlates with age-related cell loss. On the contrary, among the brain stem nuclei, the inferior olive has high lipofuscin levels and shows no age-related cell loss. Moreover, there are examples of cells that accumulate lipofuscin but do not exhibit a decline in function. This was illustrated by cell sorting experiments with cultured fibroblasts, which separated populations of cells on the basis of fluorescence intensity, and which showed that fluorescent cells suffered no loss of proliferative potential.

Thus, the evidence for antioxidant-mediated life-span extension in vertebrates does not argue persuasively for a causal role of free radicals in aging. This conclusion is generally acknowledged by advocates of the free radical theory and they offer two general explanations that could sustain the basic concept: (1) the principal site of damage (e.g., mitochondrial DNA) is not protected by exogenous antioxidants, or (2) the endogenous defense system is regulated to maintain a fixed overall level of protection so that simple attempts to increase protection by some dietary manipulation of one or a few antioxidants fail because of compensatory decreases in endogenous defenses ("compensatory downregulation"). However, the fact that lipofuscin accumulation can be reduced by feeding antioxidants in the absence of life span extension, and that tumor incidence can be reduced by feeding exogenous antioxidants, suggests that antioxidants can effectively reduce the rate of free radical damage in some tissues without undue compensatory downregulation. While compensatory downregulation would seem consistent with concepts of homeostasis, at least one study of heterozygous knockout mice with decreased MnSOD activities has shown no compensatory upregulation of other antioxidant enzymes (Table 6).

H. CELLULAR SENESCENCE

The finite doubling potential or “replicative lifespan” of cultured mammalian cells has been considered by some investigators to be a meaningful aging model. In this model normal cells usually cease to grow after about 50 population doublings regardless of the how long they are maintained in culture, a phenomenon referred to as “cellular senescence.” After growth ceases the cells remain metabolically active. Since the replicative lifespan of these cells is only dependent on the number of cell doublings and is not of time in culture it cannot be a simple function of oxidants associated with metabolism. Most studies of replicative senescence have utilized fibroblasts and reports from several laboratories have suggested a correlation of the doubling potential with the age of the cell donor.[72] However, a recent analysis has concluded that the replicative life span of cultured human fibroblasts does not correlate with donor age.[73] Despite questions about its relevance to animal aging, the cultured fibroblast model has provided some useful information about cellular effects of oxidative stress. Of particular interest are studies of the effects of oxygen tension on cell growth. Although there were conflicting initial reports[74-76] more recent studies have found an increase in the maximum number of doublings by lowering the oxygen concentration below ambient levels.[77,78] Elevated oxygen concentrations curtail both growth rates and the doubling limit, an effect that is not prevented by vitamin E supplementation.[76]

I. EXERCISE

Exercise can exert substantial effects on metabolic rate and tissue oxygen concentrations. The analysis of exercise effects requires that the intensity and duration of exercise as well as prior training be taken into account. Increased free radical production appears to occur with exhaustive exercise in untrained animals, and positive adaptations, conferring increased resistance to oxidants, are observed in endurance-trained animals. Strenuous exercise in untrained animals is associated with mechanical damage associated with muscle lengthening contractions.[79] Membrane damage under these conditions is inferred from a release of

intracellular enzymes into the bloodstream. The muscle damage elicits phagocytic activity with attendant oxidant production. The phagocytic activity increases for several days before returning to resting-state levels.[79]

There is also an immediate oxidative stress associated with exhaustive exercise, which appears to be associated primarily with the consumption of ATP. An increase in the ADP/ATP ratio, followed by AMP formation and its degradation to hypoxanthine culminates in superoxide and hydrogen peroxide production by xanthine oxidase. The importance of this pathway is demonstrated by the pronounced suppression of oxidative damage markers elicited by treating animals with allopurinol, an inhibitor of xanthine oxidase.[80] In contrast to the marked effects of tissue injury and xanthine oxidase activation, mitochondrial superoxide formation seems to be a relatively insignificant component of the damage induced by strenuous exercise in untrained animals.[80] A minimal role of mitochondrial oxidants is entirely consistent with an analysis of the mechanism of superoxide production (see sections on mitochondria and metabolism).

Evidence for an association of oxidants with exhaustive exercise has been inferred primarily from studies of post-exercise tissue samples. For example, a widely cited study of untrained rats running to exhaustion on a treadmill showed increased lipid peroxidation products in liver and skeletal muscle homogenates and in mitochondrial fractions.[81] The same study showed that plasma glutathione disulfide levels increase significantly with exhaustive exercise and that vitamin E deficiency appears to be associated with a marked decrease in endurance capacity, prompting the authors to suggest that lipid peroxidation may play a role in muscle fatigue upon exhaustion. Human studies have also suggested that oxidative stress can arise under some exercise conditions. “Extreme exercise” in healthy young men, comprising a 30 day period of 8 to 11 hours of intense exercise per day for six days per week, was reported to correlate with an increase in urinary excretion of oxidatively damaged DNA bases[82] (however, recall that this damage marker is difficult to interpret; see section on DNA damage).

The numerous reports suggesting adverse effects of exercise-induced oxidant damage have aroused skepticism, given the dramatic epidemiologic evidence of exercise benefits in humans and experimental evidence of benefits in chronically exercised animals. Female rats that have exercised throughout life have longer average life spans than their sedentary counterparts (although they do not have longer maximum life spans).[83] The mechanism of this average life-span extension is unresolved but does not seem to involve caloric restriction, since exercising animals increased their food intake to maintain peak body weights similar to those of sedentary animals. One possible factor in the beneficial effects of exercise training could be an increase the activity of antioxidant enzymes, which has been reported to occur in some animal models as well as in humans.[84] However, other work has shown no induction of SOD’s, glutathione peroxidase or change in glutathione status in human muscle tissues by exercise training.[85] Arguably, the detection of certain oxidative damage markers in tissue samples does not necessarily imply adverse health effects.

VII. DISEASE

Many diseases that shorten mean life spans are aggravated by free radical processes (see, for example, Pryor[86]). Some of the support for free radical involvement in major diseases is outlined in the following sections:

A. CANCER

The process whereby a normal cell assumes the uncontrolled growth characteristics of a cancer cell clearly involves altered genes. As summarized earlier, oxidants damage genes and therefore play a role in carcinogenesis. Genes can also be altered by non-oxidants, including alkylating and glycating agents. The literature abounds with reports of exogenous chemical and biological carcinogens. Epidemiology has established an increased risk of cancer for smoking, excessive consumption of alcohol, high levels of ionizing radiation, exposure to asbestos particles, and many other exogenous factors. Of these agents, ionizing radiation (a complete carcinogen) most directly demonstrates the carcinogenic power of free radicals. An important distinction between cancer and most other diseases or aging is that cancer generally begins with a single transformed cell. Considering the number of mitotic cells in higher animals, neoplasia is an extremely rare event, testifying to the effectiveness of the various systems that protect the genome.

Some exogenous carcinogens like vinyl chloride can be categorized by "fingerprinting" damaged DNA in terms of specific patterns of base lesions. When this fingerprinting technique was originally developed, studies of unexposed DNA showed significant background levels of base lesions expected for exogenous chemicals. It thus became apparent that analogous, or perhaps identical, chemicals were being generated endogenously.[87] Subsequent studies have shown that a host of mutagenic metabolites and molecular decomposition products arise spontaneously *in vivo*. These endogenous mutagens include not only ROS, but also aldehydes, alkylating agents, glycoxidation products, estrogen metabolites, RNS, chlorinating reagents, and δ-aminolevulinic acid (the latter is involved in heme synthesis).[87] Oxidative damage mechanisms as well as direct effects on DNA are involved in the mutagenic activity of most of these agents.

While there is abundant information about risk factors for increased cancer risk, relatively little is known about effective strategies for reducing cancer susceptibility. However, epidemiology has shown that a diet rich in fruits and vegetables correlates with lowered cancer incidence. For many years it was widely assumed that the beneficial effects of fruits and vegetables could be attributed to their content of antioxidants like vitamins C and E and carotenoids. However, it has become evident that the most potent anti-cancer agents are not simple antioxidants. By fractionating the highly effective cancer-preventing cruciferous vegetables certain chemicals (e.g., isothiocyanates) have been found to have the most potent cancer-fighting activities. Acting directly, some of these chemicals are alkylating agents. However, at appropriate doses they induce the formation of protective enzymes,[88] a phenomenon referred to as chemoprotection. As noted in the introductory section, some adaptations to stress confer broad protection against subsequent stress – possibly this could be the mechanism of action of chemoprotection.

Aging has been referred to as the most potent human carcinogen.[89] The relationship

between aging and cancer is complex; for example, neuroblastomas, many leukemias, and hormone-dependent tumors have radically different age patterns than most sarcomas and many carcinomas. The incidence of most tumors, in a large variety of species, rises dramatically with age. This rise in cancer incidence with age may be attributed to several factors:[90]

1. Long-term carcinogen exposure increases the risk of initiation, e.g., lung cancer incidence reflects duration of smoking rather than chronological age.
2. The prolonged period required for one malignant cell to multiply and develop into a detectable tumor.
3. Aging itself increases the risk because of, e.g.,:
    - a reduction in natural killer or other immune surveillance function
    - an increase in the activation of procarcinogens
    - epigenetic instability

The pathogenesis of neoplasia has been thought to be a multi-stage process comprising initiation, promotion and progression. Initiation is an irreversible alteration of gene(s) that can result from oxidative damage, promotion is a reversible process of cell proliferation that can be modulated by oxidative damage and progression is an irreversible process of de-differentiation characterized by aneuploidy and clonal variation. The process involves many variables, including tissue type, hormonal influence, proliferative rates, DNA repair capacity, environmental carcinogen exposure, viral infection, immune surveillance, and genotype. It has been suggested that cell division greatly increases the potential for non-repairable DNA damage and cancer.[91] Particularly at high doses many carcinogenic agents may exert their effects indirectly by killing cells, which stimulates the growth of new cells with concomitant DNA damage.

B. CARDIOVASCULAR DISEASE

A role of oxidant-induced damage in cardiovascular disease is supported by epidemiology studies suggesting that dietary antioxidant intake, particularly of vitamin E, correlates with a decreased incidence of heart disease.[92] Transient or prolonged occlusion of blood vessels can set the stage of the oxidant damage associated with reoxygenation of ischemic tissue a phenomenon referred to as reperfusion injury. Among the factors involved in this injury is the activity of xanthine oxidase, a source of superoxide and hydrogen peroxide. Studies with isolated heart cells (cardiomyocytes) have shown a delayed effect of hydrogen peroxide at exposure levels that caused apoptosis of only a fraction of the treated cells. This delayed response comprised a substantial increase in cell volume, consistent with the enlargement of the heart observed in some pathologies (hypertrophy).[93]

C. NEURODEGENERATIVE DISORDERS

As noted earlier, the brain would seem to be highly vulnerable to oxidative stress. Comprising about 2% of the body weight, it is responsible for about 20% of the whole-body oxygen consumption at rest. The brain contains relatively high concentrations of easily oxidizable polyunsaturated fatty acids and catecholamines, has high concentrations of H_2O_2-producing monoamine oxidase, has areas with relatively low concentrations of several

antioxidants including glutathione, vitamin E, superoxide dismutase, glutathione peroxidase and, especially, catalase (brain has about 10% of the catalase activity found in liver) as well as regions of high iron content.[94,95] Interestingly, this apparent vulnerability to oxidative stress does not cause an obvious accelerated aging of the brain relative to other tissues. As noted in the section on metabolism, this observation argues against the notion that the rate of aging is a simple function of metabolic rate. Nevertheless, aging is often associated with neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, cerebrovascular diseases (e.g., stroke) and demyelinating diseases.

Alzheimer's disease (AD) is associated with oxidative stress as seen in an elevation of several markers of oxidative damage in autopsy tissues. For example, carbonyl groups visualized by a staining procedure were found to be localized in neurofribrillary tangles (NFT), cytoplasms of neurons and nuclei of both glia and neurons.[96] Other damage markers found in AD include advanced glycation end products, nitrated tyrosine and lipid peroxidation products.[97] Iron accumulation was observed in the senile plaques and NFT that are the hallmarks of the disease and, importantly, the iron was found to be accessible to low molecular weight reducing and oxidizing agents such that it could act catalytically to produce hydroxyl radicals and oxidants derived from them.[98,99] AD may be somewhat responsive to antioxidant therapy – dietary supplementation with α-tocopherol appears to exert a slight benefit in delaying manifestations of the disease, including time of entry into nursing homes and ability to perform routine daily tasks.[100]

Parkinson's disease (PD) is a strongly age-related pathology marked by a selective and progressive loss of pigmented catecholaminergic, particularly dopaminergic, neurons of the substantia nigra pars compacta. Analyses of autopsied brain tissue have shown that advanced PD is associated with oxidized lipids, proteins and DNA consistent with a role of oxidative damage in the terminal stages of the disease.[101] A proposed model for the onset and progression of the disease suggests that oxidation of dopamine, followed by the formation of cysteine conjugates and further oxidation products leads to mitochondrial dysfunction and cell death. The model is supported by studies of the inhibitory effects of these oxidation products on the energy metabolism of isolated brain mitochondria.[102]

Other neurodegenerative diseases significantly associated with oxidative stress include multiple sclerosis, Creutzfeldt-Jacob disease and meningoencephalitis. All of these diseases are associated with significant increases in the specific and persistent lipid peroxidation marker F_2-isoprostane.[103]

D. AUTOIMMUNE DISEASE

Because tolerance to self appears to require an active thymic role in the production of T suppressor cells as well as in the deletion of self-reactive clones, one might expect thymic involution and age-related immune dysregulation to result in an age-related increase in autoimmune disease. Further, the emergence of altered self antigens through persistent viral infection, posttranslational modifications, somatic mutation, or even postmaturational development of newly expressed genes could also bring about an increased number of autoimmune reactions with age.

Autoimmune phenomena like autoantibodies, glomerulonephritis, periarteritis, and probably some classes of senile amyloid increase with age. A major role of autoimmune pathology is played in the aging of some rodents, but not all strains and species seem to be affected. Hence, while some workers have hypothesized that autoimmunity is the major aging process and likened senescence to a chronic graft-versus- host reaction, the evidence is not consistent. In humans, most known or suspected autoimmune diseases, including rheumatoid arthritis, have a peak incidence in middle age and it is difficult to assess the significance of autoimmune phenomena in human aging. Among rodents, in strains that are clearly autoimmune susceptible, such as NZB mice, antioxidant feeding has produced a delay in disease onset and life- span extension. If autoimmunity is a major aging process and not a secondary pathology, then antioxidant feeding may be said to delay the accelerated aging that has been reported in NZB mice.

Several other diseases seem to involve free radicals in their etiliology, including atherosclerosis , emphysema, arthritis, cirrhosis, and diabetes.[86]

VIII. CONCLUSIONS AND PROSPECTS

The impact of free radical damage on aging remains to be resolved (Table 8). For example, failure of conventional antioxidant supplementation to significantly extend the maximum life spans of mammals has argued against the free radical theory of aging. This failure is particularly troubling because the administration of antioxidants has clear-cut effects in reducing the extent of lipofuscin accumulation, an indication that the antioxidants do protect some cellular targets susceptible to oxidation. However, recent studies have shown that antioxidants interfere with cell signaling, which suggests possible adverse effects that could offset the intended benefits. Antioxidant administration can confer anticarcinogenic and other health benefits, which could affect mean but not necessarily maximum life spans. Recent evidence on caloric balance indicates that at least some subcellular fractions prepared from lean rodents generate fewer free radicals and are endowed with enhanced antioxidant protection relative to fractions from ad libitum-fed (obese) animals. Work on mitochondria is consistent with radical-mediated damage of mitochondrial DNA and an age-dependent dysfunction that correlates with increased free radical production and decreased antioxidant capacity. The mitochondrial data generally seem consistent with the free radical theory of aging.

Among the most exciting recent developments in aging research is the successful cloning of animals by transferring nuclei of somatic cells into enucleated oocytes. The cloning of mice to six generations, producing mice that show no signs of accelerated aging in a variety of tests, argues that the nuclear genome of at least some somatic cells suffers very little damage, oxidative or otherwise, during the lifespan of the animal.[106] On the other hand age-dependent damage to the general population of the somatic cells used for cloning is indicated by the increasing difficulty of producing viable offspring with sequential transplantations. The nuclear transfer technique is likely to be improved and will undoubtedly produce important new insights into aging mechanisms in the near future.

Another promising development is the discovery that certain enzyme-inducing constituents of vegetables and presumably other plants can confer substantial protection against cancer in animal models and, judging by epidemiologic data, in humans. Specific induction of the antioxidant response element (ARE) by phenolic antioxidants, peroxides and a variety of natural compounds, suggests strategies for lifespan extension based on manipulations of the stress response, which may be more successful in protecting against oxidative stress than dietary supplementation with simple antioxidants has been. Novel approaches to antioxidant enhancement based on synthetic agents like manganese salen compounds, nitroxides and nitrones also offer promise. Of particular interest is the role that these agents may play in cell signaling as opposed to their obvious direct effects in quenching free radicals. Body weight is receiving more attention as a risk factor in aging. Recent refinements in analyzing human mortality and morbidity statistics have led to a steady lowering of the apparent "optimal body mass index." Some recent analyses that eliminated confounding risk factors like smoking and pre-existing disease suggest that human mean life spans correlate with changes in caloric balance qualitatively similarly but quantitatively less dramatically than do those of rodents in the classic "caloric restriction" scenario.

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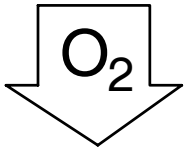
FIGURE 1. POSSIBLE INVOLVEMENT OF OXIDANTS IN AGING



Formation of Oxidants

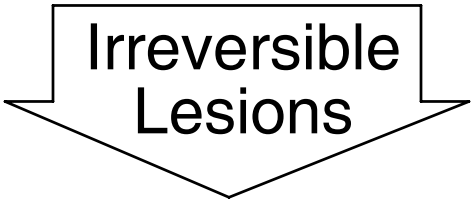
Reduction to H_2O_2	Reduction to superoxide (O_2^-)	Other oxidants
peroxisomal enzymes	mitochondrial ubisemiquinone	activated neutrophils
monoamine oxidase	xanthine oxidase	(HOCl, $NO^{\cdot}$)
xanthine oxidase	cytochrome P450	free iron, copper:
	activated neutrophils	(H_2O_2 -----> $OH^{\cdot}$)
	hemoglobin, myoglobin	ingested or inhaled toxins

Scavenging of Oxidants

H_2O_2 and ROOH	Superoxide (O_2^-)	Other oxidants
glutathione peroxidases	Cu-Zn SOD	vitamins C and E
catalase	mitochondrial Mn SOD	glutathione,
glutathione transferases		β-carotene

Cell Damage & Repair

Oxidant Targets	Protective Response
lipids	Metabolize oxiation products, *de novo* synthesis
proteins	Reductive regeneration, complete turnover
DNA	Multiple repair systems



Cumulative damage, pathology, death

Table 1. Biologic oxidants and oxidation catalysts

Name	Structure	Sources
Superoxide radical	$O_2^{\cdot-}$	Mitochondria, enzymes, some hydroquinones
Hydrogen peroxide	H_2O_2	Enzymes, superoxide
Hydroxyl radical	$OH^{\cdot}$	Hydrogen peroxide + reduced transition metal ions, peroxynitrite, superoxide + hypochlorite
Singlet oxygen	$^1O_2$	Aerobic free radical chain reactions
Nitric oxide	$NO^{\cdot}$	Nitric oxide synthase enzymes
Peroxynitrite	$ONOO^-$	Superoxide + nitric oxide
Hypochlorite	HOCl	Myeloperoxidase
Transition metal ions	Fe^{+m}, Cu^{+n}	Metalloprotein degradation, dietary overload

Table 2. Agents involved in protection against oxidants

Name	Mechanism(s)	Source (humans)
Ascorbic acid (Vitamin C)	Free radical (FR) scavenger	Dietary (see text)
Glutathione	FR scavenger, enzyme cofactor	Endogenous agent (see text)
Tocopherol (Vitamin E)	FR scavenger	Dietary (see text)
Carotenoids	Singlet oxygen scavengers	Dietary
Bilirubin	FR scavenger	Heme catabolism
Lipoic acid	FR scavenger	Endogenous agent
Uric acid	FR scavenger	Purine catabolism
Enzyme mimetic agents	Superoxide, hydrogen peroxide scavengers	Potential anti-aging drugs
PBN	FR scavenger, cell signaling effects	Potential anti-aging drug
(-) deprenyl (selegiline)	Monoamine oxidase B inhibitor	Putative neuroprotective drug
Protein methionine groups	FR scavengers	Endogenous agents
Superoxide dismutases	Superoxide scavengers	Endogenous enzymes
Catalase	Hydrogen peroxide scavenger	Endogenous enzyme
Selenium peroxidases	Hydroperoxide scavengers	Endogenous enzymes
Heme oxygenases	Heme decomposition	Endogenous enzymes
Ferritin	Iron sequestration	Endogenous enzyme
Quinone reductase	Quinone scavenging	Endogenous enzyme
Glutathione-S-transferases	Detoxification of xenobiotics, endogenous toxins, hydroperoxides	Endogenous enzymes
Peroxiredoxins	Cell signaling effects (?)	Endogenous enzymes
Metallothionine	Transition metal sequestration	Endogenous enzyme

Table 3. Production of Hydrogen Peroxide by Isolated Cell Fractions

Organelle	Oxidant Source	H_2O_2 Production (% of total)
Mitochondria	Electron transport leak	15%
Peroxisomes	Product formation	35%
Endoplasmic Reticulum	Mixed function oxidations	45%
	Xanthine oxidation	
Cytosol		5%

TABLE 4. EFFECTS OF HYDROGEN PEROXIDE ON CELLS

H_2O_2 dose	Effect on HA-1 cells
3 to 5 μM	25% to 45% growth stimulation
120 to 150 μM	Temporary growth arrest, adaptation to H_2O_2
250 to 400 μM	Permanent growth arrest without loss of function
0.5 to 1 mM	Apoptosis (nuclear condensation, DNA "laddering")
5 to 10 mM	Necrosis (membrane disruption, protein denaturation)

TABLE 5. SIGNIFICANT EXTENSIONS OF LIFE SPANS IN AGING MODELS (EXCLUDING CALORIC RESTRICTION)

Model	Intervention (Life span increase vs. wild type)	Relevance to oxidant theory of aging
Caenorhabditis elegans[48]	Nutritional SOD/catalase mimetics (44%)	Directly supportive
Caenorhabditis elegans[49]	Genetic (more than double)	Insulin response effect may be supportive
Drosophila melanogaster[50]	Genetic (Indy gene) (87-89%)	Caloric restriction analogies may provide indirect support
Saccharomyces cerevisiae[51]	Genetic (glucose mutants) (40%)	Caloric restriction analogies may provide indirect support
Mouse[52]	Genetic (deleted signal tranducer) (30%)	Supportive: lessens some H_2O_2 effects, e.g., apoptosis induction
Mouse[3]	Chronic exposure to ionizing radiation (20%)	Not supportive

Table 6. Effects of altered expression of antioxidant-related genes in mice

Genetic manipulation	Observed effect(s)	Implications for aging

MnSOD deletion	Myocardial injury, neurodegeneration, anemia, fatty liver, severe mitochondrial dysfunction, neonatal death 1-20 days after birth[53]	Supports major role of superoxide in shortening at least mean life spans
MnSOD: decreased expression in $SOD2^{-/+}$ heterozygotes	Mitochondrial dysfunction, no compensatory upregulation of other antioxidant enzymes[54]	Supports role of superoxide in mitochondrial damage
CuSOD overexpression	Cannot compensate for lethality of MnSOD loss[55]	Strongly suggests oxidative damage occurs primarily in mitochondria
GSH-Px1 deletion	Conflicting reports,[56,57] including a surprisingly consistent (but statistically insignificant) increase in life expectancy[58]	Unclear
Metallothionine overexpression or deletion	Conflicting reports[59,60]	Unclear
Heme oxygenase 1 deletion	No viable offspring, progressive chronic inflammation[61]	HO-1 is likely to modulate at least mean life spans

TABLE 7. METABOLIC POTENTIALS OF ANIMALS

(Lifetime energy expenditure in kilocalories per gram of body weight)

Species	**Metabolic Potential**
Dipteran flies	~25
Most non-primate mammals	~200
Humans	~800
Birds, bats	~1200

Table 8. Are oxidants the cause of aging? Some pro and con arguments

Pro	**Con**
Caloric restriction appears to reduce oxidative stress	Vitamin C, a superb free radical scavenger, is not synthesized by long-lived primates
Life span extension in mutants is often associated with stress resistance	Chronic ionizing radiation at low doses does not shorten lifespan, may increase it
Knockout mice lacking MnSOD or HO-1 have severely restricted survival	Dietary supplementation with natural antioxidants (vitamins C and E) does not extend life spans
Enzyme mimetics extend maximum life spans in some aging models	Tissue comparisons, e.g., brain vs. muscle, seem incompatible with simple oxidant and antioxidant models of aging
Certain drugs, PBN, (-) deprenyl, possibly acting as antioxidants have been claimed to extend life spans[104,105]	Exercise, often claimed to increase oxidant stress, exerts beneficial effects at least on mean life spans